Acid-Disintegratable Polymersomes of pH-Responsive Amphiphilic Diblock Copolymers for Intracellular Drug Delivery

Lei Wang, Guhuan Liu, Xiaorui Wang, Jinming Hu,* Guoying Zhang,* and Shiyong Liu*

CAS Key Laboratory of Soft Matter Chemistry, Hefei National Laboratory for Physical Sciences at the Microscale, iChem (Collaborative Innovation Center of Chemistry for Energy Materials), Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, Anhui 230026, China

ABSTRACT: Supramolecular vesicles, also referred to as polymersomes, self-assembled from amphiphilic polymers capable of synchronically loading with both hydrophilic and hydrophobic payloads have shown promising potential in drug delivery application. Herein, we report the fabrication of pH-responsive polymersomes via supramolecular self-assembly of amphiphilic diblock copolymers, poly(ethylene oxide)-b-poly(2-((((5-methyl-2-(2,4,6-trimethoxyphenyl)-1,3-dioxan-5-yl)methoxy)carbonyl)amino)ethyl methacrylate) (PEO-b-PTTAMA), which were synthesized via reversible addition–fragmentation chain transfer (RAFT) polymerization of a pH-responsive monomer (i.e., TTAMA) using a PEO-based macroRAFT agent. The resultant amphiphilic diblock copolymer then self-assembled into vesicles consisting of hydrophilic PEO coronas and pH-responsive hydrophobic bilayers, as confirmed by TEM and DLS measurements. The polymersomes containing cyclic benzylidene acetals in the hydrophobic bilayers were relatively stable under neutral pH, whereas they underwent hydrolysis with the liberation of hydrophobic 2,4,6-trimethoxybenzaldehyde and the simultaneous generation of hydrophilic diol moieties upon exposure to acidic pH milieu, which could be monitored by UV/vis spectroscopy, SEM, and TEM observations. By loading hydrophobic model drug (Nile red) as well as hydrophilic chemotherapeutic drug (doxorubicin hydrochloride, DOX·HCl) into the bilayer and aqueous interior of the polymersomes, the subsequent release of Nile red and DOX·HCl payloads was remarkably regulated by the solution pH values, and a lower pH value led to a faster drug release profile. In vivo experiment, observed by a confocal laser scanning microscope (CLSM), revealed that the pH-responsive polymersomes were easily taken up by HeLa cells and were primarily located in the acidic organelles after internalization, where the pH-responsive cyclic acetal moieties were hydrolyzed and the embedded payloads were therefore released, allowing for on-demand release of the encapsulants mediated by intracellular pH. In addition to small molecule chemotherapeutic drugs, biomacromolecules (alkaline phosphatase, ALP) can also be encapsulated into the aqueous lumen of the polymersomes. Significantly, the pH-triggered degradation of polymersomes could also regulate the release of encapsulated ALP, as confirmed by ALP-activated fluorogenic reaction.

INTRODUCTION

Supramolecular vesicles, also known as polymersomes, are commonly made from the self-assembly of amphiphilic block copolymers, in the core of which is an aqueous compartment enclosed by a hydrophobic bilayer membrane. Integrating both hydrophilic and hydrophobic characteristics, polymersomes are distinguished by good colloidal stability, high mechanical strength, and long circulation time compared to liposome counterparts.1-3 These unique advantages make them quite promising for drug delivery application, in which both hydrophobic and hydrophilic drugs can be simultaneously embedded.4-9 Nevertheless, although the impermeable nature of bilayers can remarkably eliminate premature drug release and minimize systemic cytotoxicity, the programmable release of encapsulants, particularly for solvated payloads in the aqueous lumen of polymersomes, is relatively difficult and uncontrollable. To resolve this issue, stimuli-responsive polymersomes, which can selectively respond to external stimuli and in turn switch the permeability of bilayers, have been explored.4,6,10,11 In this respect, a variety of stimuli including pH gradient,12-16 redox species,17-21 photoirradiation,22-25 enzymes,26,27 and magnetic and electric fields28-30 have been applied to manipulate the permeability of bilayers and to accomplish on-demand release of payloads in a spatiotemporally controlled manner.

Among all the applied triggering events, the employment of endogenous stimuli (e.g., pH gradient, redox species, and enzymes) to mediate the release profile of polymersomes, taking effect only at the desired site of action, is extremely attractive due to the overwhelming potential to optimize therapeutic efficiency and alleviate side effects. Given the various pH gradients in the physiological environment, the...
The development of pH-responsive nanocarriers in response to the mildly acidic pH of pathological regions (e.g., tumor or inflammatory tissues) as well as intracellular acidic organelles (e.g., endolysosomes) has been extensively explored. Conventional pH-labile moieties including (ortho)ester, cis-aconityl, acylhydrazone, and acetal/ketal have been used to construct pH-responsive drug carriers. In recent years, much attention has been paid to the cyclic benzylidene acetals due to their low cytotoxicity, potential to increase drug loading efficiency, and fast response to mildly acidic pH. For instance, Fréchet and co-workers developed a number of acid-degradable polymeric systems through attaching cyclic benzylidene acetals to either the backbone or the pendant chains of synthetic materials. By the copolymerization of pH-responsive acetal-based monomers, Grinstaff and co-workers fabricated pH-responsive nanoparticles and hydrogels. Zhong and co-workers developed a diversity of degradable polycarbonate polymers containing pH-sensitive cyclic acetal moieties. However, most of these pH-responsive polymeric systems composed of cyclic benzylidene acetals primarily self-assembled into spherical micelles with the capability to deliver only hydrophobic drugs. The fabrication of pH-sensitive polymersomes from cyclic benzylidene acetal derivatives is far less explored, and only a few examples are available to date. For example, Zhong et al. reported that an asymmetric triblock copolymer bearing pH-responsive cyclic benzylidene acetal moieties self-assembled into ‘chimeric’ polymersomes, which can subsequently be employed to deliver hydrophilic doxorubicin hydrochloride (DOX·HCl) and granzyme B apoptosis protein, possessing a pH-dependent release manner. Notably, albeit promising, the construction of pH-responsive polymersomes with well-defined vesicular morphology for tumor-targeted drug delivery based on pH-labile cyclic benzylidene acetals is still underdeveloped. Herein, we report a facile methodology to fabricate pH-responsive polymersomes via the self-assembly of amphiphilic diblock copolymers containing acid-sensitive cyclic benzylidene acetal moieties. A novel pH-sensitive monomer consisting of cyclic benzylidene acetal, 2-((((5-methyl-2-(2,4,6-trimethoxyphenyl)-1,3-dioxan-5-yl)methoxy)carbonyl)amino)ethyl methacrylate (TTAMA), was developed. Compared with previously reported pH-responsive monomers possessing cyclic benzylidene acetals, the conventional ester/carbonyl groups were replaced with a carbamate linkage, which was expected to provide extra hydrogen-bonding interaction and facilitate the formation of polymersomes during self-assembly process. Subsequently, well-defined amphiphilic diblock copolymers, poly(ethylene oxide)-b-TTAMA (PEO-b-TTAMA), were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization using a PEO-based macroRAFT agent. The resultant PEO-b-TTAMA diblock copolymers could self-assemble into well-defined vesicles under optimized conditions.
conditions capable of encapsulating both hydrophilic DOX-HCl and hydrophobic model drug Nile red. The pH-responsive polymersomes were relatively stable at neutral pH, thereby minimizing premature drug leakage and alleviating systemic cytotoxicity due to the impermeable nature of bilayer membranes. However, the hydrophobic bilayers were subjected to hydrolysis and degradation upon exposure to mildly acidic pH with the generation of hydrophilic diol-containing derivatives (e.g., HCAMM, Scheme 1), resulting in increased permeability of bilayers and release of loaded DOX-HCl and Nile red. Upon incubation with HeLa cells, the drug-loaded polymersomes were readily taken up by endocytosis and were mainly located in the acidic organelles (e.g., endolysosomes) after internalization, where the pH-responsive bilayers comprising cyclic benzylidene acetal moieties experienced pH-triggered disintegration. As a result, the therapeutic payloads were then released from the hydrolyzed polymersome vectors, thereby killing cancer cells in an on-demand manner (Scheme 1). Further, in order to demonstrate the generality of the pH-responsive polymersomes for controlled delivery application, the encapsulation and subsequent pH-actuated release of biomacromolecules were also explored.

**MATERIALS AND METHODS**

**Materials.** 1,1,1-Tris(hydroxymethyl)ethane (98%), 2,4,6-trimethoxybenzaldehyde (98%), dibutyltin dilaurate (DBTL), 4-methylumbelliferyl phosphate disodium salt (4-MUP), p-toluenesulfonic acid (PTSA), and doxorubicin hydrochloride (DOX-HCl) were purchased from Sinopharm Chemical Reagent Co., Ltd., and used as received. 2-Isocyanatoethyl methacrylate and Nile red were purchased from Sinopharm Chemical Reagent Co., Ltd., and used as received unless otherwise noted. PEO-based macroRAFT agent (PEO_{45}-CTA),\(^{16}\) 4-(2-methylacryloyloxyethylamino)-7-nitro-2,1,3-benzoazodiazole (NBD),\(^{16}\) and (5-methyl-2-(4,6-trimethoxyphenyl)-1,3-dioxan-5-yl)methanol precursor (1)\(^{16}\) were prepared according to literature procedures.

**Sample Preparation.** Synthetic routes employed for the preparation of TTAMA monomer (2), PEO_{45}-b-PTTAMA, diblock copolymers, BP1–BP4, via Reversible Addition–Fragmentation Chain Transfer (RAFT) Polymerization Using a Poly(ethylene oxide) (PEO)-Based MacroRAFT Agent

![Scheme 2. Synthetic Routes Employed for the Preparation of pH-Responsive TTAMA Monomer (2) and Amphiphilic PEO_{45}-b-PTTAMA, Diblock Copolymers, BP1–BP4, via Reversible Addition–Fragmentation Chain Transfer (RAFT) Polymerization Using a Poly(ethylene oxide) (PEO)-Based MacroRAFT Agent](image)

Triethylamine (TEA) and dichloromethane (CH_{2}Cl_{2}) were dried over CaH_{2} and distilled just prior to use. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., and used as received unless otherwise noted. PEO-based macroRAFT agent (PEO_{45}-CTA),\(^{16}\) 4-(2-methylacryloyloxyethylamino)-7-nitro-2,1,3-benzoazodiazole (NBD),\(^{16}\) and (5-methyl-2-(4,6-trimethoxyphenyl)-1,3-dioxan-5-yl)methanol precursor (1)\(^{16}\) were prepared according to literature procedures.

**Synthesis of TTAMA Monomer (2, Scheme 2).** In a typical experiment, the cyclic acetal precursor 1 (1 g, 3.4 mmol) and dibutyltin dilaurate (DBTL, 100 μL) were dissolved in anhydrous toluene (25 mL) and then azetroptic distillation was carried out under reduced pressure at 50 °C to remove most of the toluene. 2-Isocyanatoethyl methacrylate (0.79 g, 5.1 mmol) was then added dropwise. After stirring at 35 °C overnight, the solvents were removed under reduced pressure via a rotary evaporation. The crude product was further purified by column chromatography, affording as a pale yellowish solid (1.45 g, 3.2 mmol, yield: 94%).\(^{1}\)H NMR (CDCl_{3}, δ, ppm, Figure S1a): 6.12 (d, J = 5.7 Hz, 1H), 6.10 (d, J = 5.3 Hz, 2H), 5.96 (s, 1H), 5.59 (d, J = 1.4 Hz, 1H), 5.02 (s, 1H), 4.57 (s, 2H), 4.23 (t, J = 5.3 Hz, 2H), 4.02 (d, J = 11.7 Hz, 2H), 3.80 (d, J = 11.9 Hz, 9H), 3.67–3.54 (m, 2H), 3.54–3.39 (m, 2H), 1.95 (s, 3H), 0.78 (s, 3H).\(^{13}\)C NMR (CDCl_{3}, δ, ppm, Figure S1b): 16.85, 18.28, 34.06, 40.12, 55.23, 55.86, 63.71, 67.4, 73.61, 90.84, 96.89, 106.99, 125.0, 135.94, 156.72, 159.64, 161.84, 167.25. ESI-MS (m/z, Figure S2): [M⁻]: calcd for C_{22}H_{31}NO_{9}: 454.20; found: 454.206.

**Synthesis of PEO_{45}-b-PTTAMA, Amphiphilic Diblock Copolymers (Scheme 2).** The PEO_{45}-b-PTTAMA, diblock copolymers (BP1–BP3) with different PTTAMA block lengths were synthesized via RAFT polymerization of TTAMA monomer using a PEO-based macroRAFT agent. Using the preparation of BP2 diblock copolymer as an example, in a typical experiment, TTAMA (2, 0.478 g, 1.05 mmol), PEO_{45}-CTA (0.06 g, 0.03 mmol), AIBN (1.0 mg, 0.006 mmol), and dry THF (1.2 mL) were added into a polymerization tube equipped with a magnetic stirring bar. The tube was carefully degassed by three freeze–pump–thaw cycles and then sealed under vacuum. After being thermostated at 70 °C in an oil bath and stirred for 12 h,
the reaction tube was quenched into liquid nitrogen, opened, and
diluted with THF. The mixture was then precipitated into an excess
amount of diethyl ether three times, and the final BP2 copolymer
was obtained as yellowish powder after being dried in a vacuum oven at
room temperature overnight. The degree of polymerization (DP) of
the PTTAMA block was determined to be ~25 by 1H NMR analysis
(Table 1 and Figure S3). Thus, the BP2 diblock copolymer was
denoted as PEO_b-b-PTTAMA_{15}.

BP1 and BP3 diblock copolymers and NBD-labeled diblock
copolymer, BP4, were also synthesized according to a similar protocol.
The structural parameters of the resulting diblock copolymers are
summarized in Table 1.

### Table 1. Molecular Parameters of PH-Responsive Amphiphilic Diblock Copolymers Used in This Study

<table>
<thead>
<tr>
<th>entry</th>
<th>samples</th>
<th>$M_c^c$ (kDa)</th>
<th>$M_c/M_c^c$</th>
<th>⟨$D_2$⟩ (nm)</th>
<th>$\mu_s/T_1^{CC}$</th>
<th>morphology$^d$</th>
</tr>
</thead>
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<tr>
<td>BP1</td>
<td>PEO_{45}-b-PTTAMA_{15}</td>
<td>8.1</td>
<td>1.19</td>
<td>140</td>
<td>0.069</td>
<td>M</td>
</tr>
<tr>
<td>BP2</td>
<td>PEO_{45}-b-PTTAMA_{15}</td>
<td>13.6</td>
<td>1.29</td>
<td>680</td>
<td>0.338</td>
<td>V</td>
</tr>
<tr>
<td>BP3</td>
<td>PEO_{45}-b-PTTAMA_{15}</td>
<td>27.2</td>
<td>1.38</td>
<td>190</td>
<td>0.183</td>
<td>M, V</td>
</tr>
<tr>
<td>BP4</td>
<td>PEO_{45}-b-PTTAMA_{25}</td>
<td>13.5</td>
<td>1.23</td>
<td>700</td>
<td>0.283</td>
<td>V</td>
</tr>
</tbody>
</table>

$^a$Calculated from $^1$H NMR analysis. $^b$Determined by GPC using THF as the eluent. $^c$Determined by dynamic light scattering (DLS).

$^d$Determined by TEM observations (M = micelles; V = vesicles).

dissolved in 2 mL of disodium hydrogen phosphate/sodium citrate
buffer (pH 7.4, 10 mM) was slowly added into the THF solution of
BP2 diblock copolymer within 1 h, followed by the injection of 7 mL
of buffer solution within 1 h. After being stirred for another 2 h, THF
and unloaded DOX·HCl were removed by dialysis (MWCO 3.5 kDa)
against buffer solution (pH 7.4). According to a standard calibration
curve, the DOX·HCl loading efficiency (LE) was determined to be
25.3% and the DOX loading content (LC) was appropriately 5.9 wt
%. Subsequently, 100 μL aqueous dispersion of DOX-loaded BP2
polymersomes (1.0 mg/mL) was transferred to a dialysis cell with a
molecular weight cutoff (MWCO) of 2.0 kDa and then dialyzed against
3.4 mL of disodium hydrogen phosphate/sodium citrate buffer
(pH 4.5, 5.0, 6.0, and 7.4) for 37 °C. The DOX·HCl concentrations in
the dialysate were quantified by measuring the fluorescence intensity
of DOX·HCl at 595 nm (λ_ex = 480 nm) against a standard calibration
curve.

### Intracellular Uptake of Polymersomes Observed by Confocal Laser Scanning Microscopy (CLSM).

The cellular uptake of polymersomes was studied toward human epithelioid cervix carcinoma (HeLa) cells using fluorescence microscopy. HeLa cells were seeded onto glass coverslips in a four-well culture plate at a density of 1 × 10^4 cells/well. After 24 h culture, the cells were treated with 20 μL of BP4
polymersomes (1 mg/mL) for 4, 8, and 12 h. DAPI (0.5 μL) and
Lysotracker red (0.5 μL) were employed to stain nuclei and
endolysosomes, respectively. After incubation, the cell monolayers
were rinsed three times with 1 mL of PBS (10 mM, pH 7.4) to remove
BP4 polymersomes. Fresh PBS (10 mM, pH 7.4) was added to the
plates, and the cells were observed and imaged under a confocal laser
scanning microscope.

### Intracellular Acidic pH-Actuated DOX·HCl Release from Internalized Polymersomes.

HeLa cells were seeded onto glass coverslips in a four-well culture plate at a density of 1 × 10^4 cells/well. After 24 h
incubation, the HeLa cells were treated with DOX-loaded polymersomes (1 μg/mL, equivalent DOX concentration) for
predetermined time (8 h). Then the cell monolayers were rinsed three times with 1 mL of PBS (10 mM, pH 7.4) to remove
either polymersomes. After that fresh PBS (10 mM, pH 7.4) was added to the plates, and the cells were further incubated for a predetermined time. DAPI was used to stain the nuclei, and the cells were observed and imaged. Similarly, for time-dependent cellular uptake studies of
DOX-loaded polymersomes, the glass coverslips were removed from the incubator at predetermined time intervals, and the cells were
processed using the above confocal studies protocol.

### In Vitro Cytotoxicity Assessment.

Cell viability was examined by the MTT assay. Briefly, HeLa cells were seeded in a 96-well plate at the initial density of 8000 cells/well in 100 μL of DMEM complete medium. Free and drug-loaded polymersomes were then added to achieve varying final polymer concentrations. After incubation for 48 h, MTT reagent (20 μL of PBS buffer, 5 mg/mL) was added to each well, and the cells were further incubated with 5% CO₂ for 4 h at 37 °C. The culture medium in each well was removed and replaced by 100 μL of DMSO. The solution from each well was transferred to another 96-well plate, and the absorbance values were recorded at the wavelength of 490 nm using a microplate reader (Thermo Fisher).

Each experiment was done in quintuple. The data are shown as the mean value plus a standard deviation (±SD).

### pH-Triggered Degradation of Polymersomes and Release of Encapsulated Enzymes Probed by Enzymatic Fluorogenic Reaction.

ALP-loaded BP2 polymersomes were first prepared via a similar self-
assembly procedure. Briefly, BP2 diblock copolymer (2 mg) was dissolved in 1 mL of THF, and the solution was stirred and maintained at 25 °C in a water bath for 20 min. Then, the aqueous solution of ALP (1 mg, 2 mL) was slowly injected within 1 h, followed by the injection of 7 mL water within 1 h. After being stirred for another 2 h, THF and free ALP were removed by dialysis against pure water and ultrafiltration−centrifugation (Millipore, MWCO 100 kDa), respectively.

Subsequently, the as-prepared ALP-loaded BP2 polymersome solution was equally divided into four batches, and each test tube contained 2.5 mL of BP2 polymersome solution. The pH values of three tubes were adjusted to 5.0, and the last one was adjusted to 7.4 by addition of disodium hydrogen phosphate/sodium citrate buffer (10 mM). After being stirred at room temperature for different time intervals, the solution pH values were adjusted to 7.4 via addition of disodium hydrogen phosphate/sodium citrate buffer (0.1 M), and the final volume of each solution was adjusted to 5 mL by adding deionized water. Finally, each of the solutions was further equally divided into two groups. One of the batches was ultrafiltration−centrifuged and the other one left untreated. After that, 4-MUP was divided into two groups. One of the batches was ultrafiltration−centrifugation (Millipore, MWCO 100 kDa), respectively.

RESULTS AND DISCUSSION

Cyclic benzylidene acetal moieties have been extensively employed for the construction of pH-sensitive drug nanocarriers.65−67 These acid-degradable moieties can be selectively attached to the backbones, pendant chains, and distal ends of polymers through rational chemical designs. However, despite possessing unique acid-responsive characteristic, these pH-responsive polymers typically self-assembled into spherical nanoparticles.48,53,68 Aiming to facilitate the formation of other morphologies (e.g., polymersomes) and regulate the drug-loading and releasing efficiency, we attempted to introduce additional hydrogen-bonding interaction in the pendant chains via the incorporation of carbamate linkages instead of conventional ester/carbonate bonds, which has been confirmed to be effective on the fabrication of photosensitive polymersomes in our recent work.23 We surmised that the additional hydrogen bonding interaction, together with the hydrophobic association originated from pH-responsive cyclic benzylidene moieties, could cooperatively facilitate the formation of vesicular assemblies. To this end, TTAMA monomer (2) composed of an acidic-responsive cyclic benzylidene moiety and a carbamate linkage was synthesized via the condensation of hydroxyl-containing cyclic benzylidene acetal precursor (1) and 2-isocyanatoethyl methacrylate in the presence of DBTL catalyst (Scheme 2). The chemical structure
of TTAMA monomer was confirmed by $^1$H and $^{13}$C NMR analysis and ESI-MS spectrum (Figures S1 and S2).

Subsequently, pH-responsive amphiphilic diblock copolymers with varying DPs of PTPTAMA blocks, PEO$_{45}$−b−PTPTAMA$_{n}$ (BP1−BP3, Table 1), were synthesized via RAFT polymerization of TTAMA monomer using a PEO-based macroRAFT agent (Scheme 2). The DPs of the resulting diblock copolymers were calculated from $^1$H NMR spectroscopy, and the typical $^1$H NMR spectrum of PEO$_{45}$−b−PTPTAMA$_{25}$ copolymer (BP2) is shown in Figure S3, while GPC elution profiles shown in Figure S4 revealed the formation of well-defined diblock copolymers with relatively narrow polydispersity indexes ($M_w/M_n < 1.4$, Table 1). The structural parameters of the as-synthesized diblock copolymers are summarized in Table 1. In addition, green-emissive dye (NBD)-labeled diblock copolymer BP4 was also synthesized according to a similar procedure via the RAFT copolymerization of TTAMA and NBD monomers, and its structural parameters are included in Table 1 as well.

It is well-documented that amphiphilic diblock copolymers can self-assemble into numerous morphologies in selective solvents such as spherical micelles, nanorods, lamellas, and vesicles, highly relying on the hydrophilicity/hydrophobicity ratios of the copolymer compositions and other parameters including initial polymer concentration, water addition rate, and the cosolvent used. In the next step, the self-assembly behavior of BP1−BP3 diblock copolymers was investigated and the as-assembled morphologies of BP1−BP3 were observed by TEM and SEM. Using THF as a cosolvent and at a fixed water addition rate of 4 mL/h, BP1−BP3 copolymers self-assembled into spherical micelles, vesicles, and a mixture of micelles and vesicles, respectively (Figure 1 and Table 1). The corresponding intensity average hydrodynamic diameters, $\langle D_h \rangle$, were 140, 680, and 190 nm, as determined by DLS measurements (Table 1 and Figure 1d). We inferred that the distinct morphologies with varying diameters could be ascribed to the differences in the hydrophobic PTPTAMA block lengths and the extra contribution of the hydrogen bonding interaction originating from the carbamate linkages in the pendant chains. Moreover, we found that the final $\langle D_h \rangle$ was remarkably affected by the water addition rate. For example, although BP2 diblock copolymer can self-assemble into vesicular nanostructures at the water addition rates ranging from 0.5 to 4.0 mL/h, the $\langle D_h \rangle$ of resultant vesicles gradually decreased when increasing the water addition rate (Figure S5). We speculated that at a higher water addition rate the hydrophobic PTPTAMA blocks were frozen more quickly under a progressive water addition process, leading to the formation of vesicles with lower $\langle D_h \rangle$ due to the lack of enough time to relax and rearrange the polymer chains.

Given the pH-responsive feature of the cyclic benzylidene acetal moieties, in the following step, we investigated the pH-triggered hydrolysis of the polymersomes through the combination of a number of techniques including DLS, UV−vis spectroscopy, SEM, TEM, and $^1$H NMR spectroscopy. Upon incubating the BP2 polymersomes in an acidic pH buffer (e.g., pH 5.0) for 36 h, the $\langle D_h \rangle$ of polymersomes initially increased within the first 8 h and the unimodal distributions of hydrodynamic diameters remained unchanged, indicating the gradually swelling of polymersomes. Further, after 36 h incubation, a bimodal rather than unimodal distribution of the hydrodynamic diameter of hydrolyzed polymersomes was observed, and the polydispersity index drastically increased from 0.338 to 0.738 (Figure 2a), presumably due to the formation of hydrophilic diol derivatives resulting from the hydrolysis of the cyclic benzylidene acetal. In contrast, no significant change in $\langle D_h \rangle$ was observed for the polymersomes solution incubated at pH 7.4 (Figures 2b), and there was no compromise in the stability of as-assembled polymersomes in serum at pH 7.4 after 24 h incubation, in accord with the acid-responsive nature of the cyclic benzylidene acetal moieties. Moreover, the pH-dependent hydrolysis of BP2 polymersomes
was also manifested by the steady drop in scattering intensity, revealing that a lower pH value led to a more prominent decrease within the same incubation period, while negligible change in scattering intensity was detected at pH 7.4, in line with the pH-dependent changes in $\langle D_h \rangle$ (Figure 2c). The release of 2,4,6-trimethoxybenzaldehyde residues as a result of the pH-triggered cleavage of the cyclic benzylidene acetamoieties was monitored by UV/vis spectroscopy at the wavelength of 292 nm, corresponding to the absorbance of released 2,4,6-trimethoxybenzaldehyde, and a lower pH was more beneficial for rapid degradation of the acid-responsive cyclic acetals (Figure 2d). Specifically, the BP2 polymersomes underwent $\sim 77\%$ hydrolysis after 72 h of incubation at pH 5.0, and a complete hydrolysis was achieved within the same incubation time at pH 4.5, whereas less than 10% hydrolysis was observed at pH 7.4 after 72 h incubation (Figure 2d).

Additionally, the pH-triggered degradation of BP2 polymersomes was further corroborated by TEM and SEM observations, revealing the gradual disruption and disappearance of vesicular structures after incubation at pH 5.0 (Figure 3), consistent with the appearance of bimodal distribution of hydrodynamic diameter (Figure 2a). Upon hydrolysis for 5 days incubation at pH 5.0, the $^1$H NMR spectrum of recovered hydrolyzed BP2 diblock copolymer revealed an 82% hydrolysis degree, and the corresponding TEM image revealed the presence of spherical nanoparticles due to an incomplete hydrolysis (Figure S6). Notably, the previously reported drug delivery vehicles containing cyclic benzylidene acetals typically underwent full degradation within 72 h at pH 5.0,54,56,59 the current polymersomes exhibiting a relatively slow hydrolysis kinetics could be attributed to the presence of hydrogen bonding from carbamate linkages in the pendant chains, which impeded the diffusion of protons and thus slowed down the hydrolysis process. As a result, the hydrolysis kinetics of the pH-responsive polymersomes can be facilely tuned by not only pH values applied but also by specific noncovalent interactions within the supramolecular assemblies (hydrogen bonding in this case), which may be advantageous for extending the drug release time.

In comparison with micelle-based drug delivery systems with only hydrophobic domains, polymersomes with vesicular structures possess unique advantage since both hydrophilic and hydrophobic drugs can be encapsulated simultaneously.5,6 Subsequently, we engineered the pH-responsive polymersomes as a smart drug delivery vehicle and the intracellular pH gradient was employed as the triggering event to regulate the release of encapsulants.73,74 As expected, both hydrophilic anticancer drug, DOX·HCl, and hydrophobic model drug, Nile red, could be embedded into the aqueous lumen and the hydrophobic bilayer of the polymersomes, respectively. The pH-induced release of loaded DOX·HCl and Nile red was monitored by the evolution of the corresponding fluorescence intensities recorded at 598 and 625 nm for DOX and Nile red, respectively (Figure 4, Figures S7 and S8). The fluorescence intensities of both DOX·HCl and Nile red experienced negligible changes upon being incubated at pH 7.4, in line

Figure 3. (a–c) TEM images and (d–f) corresponding SEM images recorded for BP2 polymersomes (a, d) before and (b, c, e, f) after being hydrolyzed at pH 5.0 for (b, e) 24 h and (c, f) 48 h.

Figure 4. (a) Schematic representation of pH-triggered corelease of hydrophobic Nile red and hydrophilic DOX·HCl from BP2 polymersomes. Upon decreasing solution pH, the hydrophobic bilayer was subjected to hydrolysis and became hydrophilic and permeable, leading to corelease of DOX·HCl and Nile red. Release profiles of (b) Nile red from the hydrophobic bilayers and (c) DOX·HCl from the aqueous interiors of polymersomes at varying pH. Results are presented as the means ± SD in quintuplicate.
with the impermeable nature of the bilayers under neutral pH milieu. However, under mildly acidic conditions (pH 4.5, 5.0, and 6.0), the fluorescence of Nile red underwent progressive drops, while the DOX·HCl fluorescence in dialysate exhibited steady rises (Figures S7 and S8), manifesting the corelease of DOX and Nile red resulted from the acid-activated disruption of the polymersomes. Further, the release rates of both DOX·HCl and Nile red were highly pH-dependent, which were accelerated upon decreasing the solution pH values (Figure 4b,c), in consistence with the pH-dependent degradation performance of BP2 polymersomes (Figure 2).

It should be noted that this pH-dependent release manner is favorable for eradicating premature drug release before arriving at the target regions of action and thus alleviating the systemic cytotoxicity for drug delivery application. In the next phase of work, we investigated the intracellular drug delivery performance based on the pH-responsive polymerosome vector. In order to trace the intracellular trafficking of the pH-responsive polymersomes, green emissive dye, NBD, was incorporated into the pH-responsive PTTAMa block, affording PEO_{4.6-b-P-}

\[
\text{TTAM}_{0.997-cyco-NBD}_{0.003} \text{BP}_4 \quad (\text{BP}_4, \text{Table 1}).
\]

Upon incubation HeLa cells with BP4 polymersomes, the green emission from NBD dye gradually increased within 12 h incubation time (Figure S9), suggesting an efficient internalization of BP4 polymersomes. Meanwhile, the colocalization ratio between the green channel of NBD and the red channel of LysoTracker red quantified from the CLSM images steadily increased from ~43% at 4 h incubation to ~77.4% at 12 h incubation (Figure S9b), demonstrating that the internalized BP4 polymersomes were mainly located in the acidic endolysosomes. Given the pH-responsive nature of the polymersomes, this preliminary result should be quite promising for the pH-mediated intracellular drug release and encouraged us to explore the potential of the polymersomes as a novel drug delivery carrier.

Building on the above results, in the following step, the intracellular drug delivery capability of the pH-responsive polymersomes was further investigated by assessing the therapeutic performance of the DOX-loaded polymersomes in inhibiting the proliferation of HeLa cells. As shown in Figure 5, after 8 h incubation, the red emission of DOX·HCl overlapped quite well with the green emission of BP4 vector, and there was no appreciable red emission in the nuclei and cytosols, suggesting that the DOX·HCl was initially located in the aqueous lumen of BP4 polymersomes. However, upon extending the incubation time to 48 h, DOX·HCl payload was gradually released from the polymersomes and subsequently entered into the nuclei (Figure 5). This process was accompanied by the decreased colocalization ratio between the green channel of NBD and the red channel of DOX and the concurrently increased colocalization ratio between the blue channel of DAPI and the red channel of DOX (Figure S10). We reasoned this result was the disintegration of polymersomes in the acidic endolysosomes, enabling the release of encapsulated DOX·HCl.

In vitro cytotoxicity assay demonstrated that the BP2 polymersomes without DOX loading were nontoxic up to a concentration of 1.0 g/L and over 80% of HeLa cells survived after 48 h of incubation (Figure 6a). Nevertheless, the DOX-loaded polymersomes displayed significantly increased cytotoxicity, comparable to that of free DOX·HCl, in agreement with the previous reports (Figure 6b). Specifically, less than 30% of cells survived at an equivalent DOX·HCl concentration of 5 \( \mu g/mL \), and the half-inhibitory concentration (IC_{50}) of DOX-loaded polymersomes was determined to be 0.73 \( \mu g/mL \). The significantly increased cytotoxicity of DOX-loaded polymersomes over DOX-free polymersomes should be a result of the acidic-\pH triggered disintegration of the polymersomes, followed by the release of loaded DOX·HCl within the cells.

As confirmed above, the cyclic benzylidene acetal moieties embedded within the polymerosome bilayers underwent pH-triggered hydrolysis, and this unique pH-responsive characteristic can be successfully used for the fabrication of intracellular drug delivery vector. To further demonstrate the versatility of pH-activated degradation of the polymersomes that regulated the release of payloads, biorelevant macromolecules (e.g., enzyme) rather than small molecule chemotherapeutic drugs were encapsulated into the aqueous lumen of polymersomes, and the pH-triggered release profile of biomacromolecules was studied (Figure 7). According to a standard calibration curve (Figure S11b), the ALP loading content was determined to be 35.6 wt %. Alkaline phosphatase (ALP) that can selectively hydrolyze phosphate groups was initially loaded into the
aqueous lumen of BP2 polymersomes during the self-assembly process. Under neutral condition (pH 7.4), the subsequent introduction of 4-MUP, an enzymatic substance of ALP, into the BP2 polymersome solution resulted in no substantial fluorescence turn-on due to the poor permeability of bilayers that segregated the ALP and 4-MUP substance. In sharp contrast, upon introducing 4-MUP substance into the polymersome solutions after incubation at pH 5.0, pronounced fluorescence emission of the decaged 4-MUP (i.e., 7-hydroxyl-4-methylcoumarin, HMC) was discerned, which increased linearly as a function of the incubation time (Figure 7b). This result clearly suggested that after being treated under acidic pH (e.g., 5.0), ALP and 4-MUP could encounter with each other in the solution due to the enhanced permeability of the bilayers. The enzymatic dephosphorylation reaction could therefore be activated, resulting in fluorescence turn-on.

Considering the negligible fluorescence changes at pH 7.4, we surmised that the acid-triggered degradation of pH-responsive cyclic benzylidene acetal moieties and the resulting generation of hydrophilic diol groups should account for the increased permeability, allowing for the activation of ALP-mediated dephosphorylation reaction of 4-MUP. It should be noted that the emerging fluorescence of HMC moieties, after incubation in acidic milieu, could come from two synergistic contributions: (1) 4-MUP substance diffused into the polymersomes and then reacted with ALP; (2) ALP enzyme diffused out from the aqueous lumen of polymersomes and then activated 4-MUP outside the polymersomes. To clarify whether the encapsulated ALP was released from the degraded polymersomes, the polymersome solutions after incubation at pH 5.0 were further treated with ultrafiltration and 4-MUP substance was further treated with the supernatant and unfiltered solution, respectively. Notably, since the fluorescence of 4-MUP can be selectively activated by ALP and there was a linear relationship between the ALP amount and fluorescence intensity change rates of 4-MUP in the investigated concentration range (Figure S11b), the fluorescence intensity of 4-MUP can in turn indicate the relative concentrations of ALP. Consequently, the released ALP contents in the supernatant from polymersomes subjected to acidic disintegration could be defined as the fluorescence intensity ratio of HMC moieties: \[ \text{released ALP} \% = \frac{I_{\text{HMC,filtrate}}}{I_{\text{HMC,unfiltered solution}}} \times 100\% \] (1)

where \( I_{\text{HMC,filtrate}} \) is the fluorescence intensity of HMC activated by the supernatant of polymersomes after ultrafiltration subjected to hydrolysis at pH 5.0 at predetermined time and \( I_{\text{HMC,unfiltered solution}} \) is the fluorescence intensity of HMC activated by the unfiltered polymersome solution at pH 5.0 with otherwise the same conditions. As shown in Figure 7c, the fluorescence intensity ratio of HMC \((I_{\text{HMC,filtrate}} / I_{\text{HMC,unfiltered solution}})\) monotonously increased from 40% after 24 h incubation to ∼90% after 5 days incubation, clearly indicating that the encapsulated ALP enzyme was gradually released from the lumen of polymersomes after incubation under acidic pH. In contrast, no noticeable change in the fluorescence ratio was observed after being incubated at pH 7.4 for the same time period. In addition, this result demonstrated that the pH-responsive polymersomes could also be employed to deliver therapeutic biomacromolecules (e.g., proteins and vaccines), exhibiting pH-responsive release profile. Taken together, not only small molecule chemotherapeutic drugs but also biomacromolecules could be incorporated into the polymersomes to fabricate pH-responsive drug delivery vehicles, and the controlled release of encapsulants could be regulated by taking advantage of pH-triggered degradation of polymersomes and thus enhanced permeability of bilayers.
CONCLUSION

In summary, a novel pH-responsive monomer, TTAMA, comprising a pH-responsive cyclic benzylidene acetal moiety and a carbamate linkage in the pendant chain was successfully prepared, and thereafter corresponding amphiphilic diblock copolymers, PEO-b-PTTAMA, were synthesized via RAFT polymerization using a PEO-based macroRAFT agent. The as-synthesized amphiphilic diblock copolymer facilely self-assembled into well-defined polymersomes, presumably due to the cooperative hydrophobic association and hydrogen bonding interaction. Interestingly, the self-assembled morphology can be tuned by both the polymer composition and water addition rate. Because of the incorporation of pH-responsive cyclic benzylidene acetal groups, the polymersomes were distinguished by unique pH-responsive characteristic. Albeit relatively stable under neutral pH, the polymersomes were subjected to pH-triggered hydrolysis with the release of 2,4,6-trimethoxybenzaldehyde and the resultant generation of hydrophilic diol derivatives under mildly acidic pH, corresponding to the pH in endolysosomes. The as-assembled polymersomes were capable of loading both hydrophilic drug in the aqueous interiors and hydrophobic drugs in the bilayers. In vitro study demonstrated that the polymersomes could be efficiently taken up by HeLa cells and principally accumulated into the acidic organelles, where pH-responsive acetal moieties were gradually degraded, resulting in enhanced permeability of bilayers and the release of loaded drugs and killing cancer cells. Further, we demonstrated that not only small molecule drugs but hydrophilic biomacromolecules (e.g., enzyme) can also be encapsulated into the aqueous interior of polymersomes, and the release of biomacromolecules manifested a pH-dependent manner. The utilization of intracellular pH as the triggering event for the controlled release of encapsulants from polymersomes represents a promising strategy for the efficient treatment of cancers, in which combinational therapeutic drugs (small and big molecule drugs) could be synergistically incorporated to improve clinical efficacy.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.5b01709.

Additional characterization data including NMR spectroscopy, GPC elution profile, TEM, DLS, fluorescence spectra, and confocal images (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: slu@ustc.edu.cn (S.L.).
*E-mail: hjm85@mail.ustc.edu.cn (J.H.).
*E-mail: gyzhang@ustc.edu.cn (G.Z.).

Notes

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